Available online at www.sciencedirect.com
ScienceDirect
Journal of Forensic and Legal Medicine 15 (2008) 368–372
JOURNAL OF FORENSIC AND LEGAL MEDICINE
www.elsevier.com/jflm

Original Communication

Immunohistochemical analysis of P-Selectin as a possible marker of vitality in human cutaneous wounds ☆,☆☆

J.A. Ortiz-Rey MD [a,*], J.M. Suárez-Peñaranda MD PhD [b], P. San Miguel MD PhD [a], J.I. Muñoz MD PhD [b], M.S. Rodríguez-Calvo MD PhD [b], L. Concheiro MD PhD [b]

[a] *Department of Pathology, POVISA Hospital, Vigo, Spain*
[b] *Department of Pathology and Forensic Sciences, University of Santiago de Compostela, Spain*

Received 22 November 2007; received in revised form 15 January 2008; accepted 8 February 2008
Available online 13 June 2008

Abstract

Immunohistochemical detection of mediators of inflammation, such as P-Selectin, has been proposed to assess vitality of wounds. Forty-five incised cutaneous wounds (24 vital, 14 post-mortem, seven with induced autolysis/putrefaction) were immunostained with antibodies against P-Selectin and CD31. The percentage of stained lumina for P-Selectin out of the total of CD31 positive vessels (P-S/CD31 index) was calculated at both edges of every specimen. In vital samples, the P-S/CD31 index ranged from 10.7% to 71.4% at the wound edge, and was 12.5–58.8% for the opposite margin, with a ratio between both indices of 0.37–1.77 (mean: 0.94). In post-mortem cases, the index ranged from 22.5% to 69.2% at the wound edge, and was 28–89.5% at the opposite margin, with a ratio between both indices of 0.76–1.9 (mean: 0.96). Differences between ratios were not statistically significant and thus precluded any assessment of vitality. The analysis of P-Selectin/CD31 immunoreactivity in skin wounds was not useful for the diagnosis of vitality when evaluating both edges for each specimen. Moreover, P-Selectin has been detected in post-mortem injuries and it is not specific to vital injuries. Microscopic evaluation becomes difficult after autolysis/putrefaction.
© 2008 Elsevier Ltd and FFLM. All rights reserved.

Keywords: Forensic pathology; Vital reaction; Immunohistochemistry; Wound healing; Selectin

1. Introduction

The determination of the vitality of skin wounds constitutes a common problem for the forensic pathologist. Differential diagnosis between vital and post-mortem wounds is based upon gross and routine histological studies, complemented by selected ancillary techniques such as biochemical methods and immunohistochemistry.[1,2]

The assessment of vitality becomes especially difficult when the injury was inflicted very close to the time of death. The immunohistochemical analysis of factors that mediate initial events of the inflammatory reaction might be useful on such purpose.[2,3]

Fibronectin and P-Selectin are worthy of note and have been proposed as extremely early immunohistochemical markers of vitality in skin injuries.[4–7]

Fibronectin is an extra-cellular matrix glycoprotein that rapidly accumulates in the dermis soon after the injury and probably derived from damaged blood vessels.[8] The overexpression of fibronectin has been described in vital wounds aged as little as a few minutes.[5,6] However, the sensitivity as well as the specificity of this marker has been questioned in some reports.[8–10]

P-Selectin is a 140 kDa transmembrane glycoprotein present in megakaryocytes, activated platelets and activated

☆ Supported in part by Grant PGIDT01PXI20807PR.
☆☆ Presented in part at the 17th Meeting of the International Association of Forensic Sciences, Hong Kong, August 21–26, 2005.
* Corresponding author. Address: Servicio de Anatomía Patológica, Hospital POVISA, Salamanca 5, 36211 Vigo, Spain. Tel.: +34 986 413 144; fax: +34 986 421 439.
E-mail address: jaortiz@povisa.es (J.A. Ortiz-Rey).

1752-928X/$ - see front matter © 2008 Elsevier Ltd and FFLM. All rights reserved.
doi:10.1016/j.jflm.2008.02.011

endothelial cells. In non stimulated platelets and endothelial cells, it is stored in the membranes of secretory granules (classically named Weibel-Palade's bodies in the endothelium), and they are not detected on the cell surface. When platelets or the endothelium are activated, the granules fuse with the cellular membrane and P-Selectin is expressed on the surface.[11–13] P-Selectin contributes to promote leukocyte rolling, which is one of the first cellular events in an inflammatory reaction.[14,15]

To the best of our knowledge, few papers have researched the value of P-Selectin as a marker of vitality, and those that did compared wound specimens with control samples obtained from the same bodies.[7,16] We have used a different approach analysing specifically wound specimens and not undamaged skin.

Our objective has been to investigate the expression of P-Selectin in single vital and post-mortem wounds in order to evaluate the sensitivity and specificity of this method to assess vitality.

2. Methods

The study comprised 45 samples from human incised cutaneous wounds:

- Twenty-four vital injuries, obtained from surgical incisions in 13 men and 11 women with an average age of 57.1 years (range: 35–76). The resulting wounds were aged from 10 to 260 min (mean: 116.1).
- Fourteen post-mortem samples, obtained from autopsy abdominal incisions in nine men and five women aged 31–82 years (mean: 58.7). The time between death and necropsy was 1.5 to 16 h (mean: 6), during which time corpses were maintained under refrigeration. The wounds were collected from the bodies at 15–180 min post-infliction (mean: 97.8).
- Seven samples with an induced artifact of delayed fixation: these were left at room temperature in a saline solution for 1–63 days (mean: 25.3) before being fixed.

Injuries had been inflicted with a scalpel to a dept of 1.5 cm in the abdominal skin. Transversal sections were taken from the wounds, marking the non-wound edge with ink.

Specimens were routinely fixed in 4% formaldehyde and embedded in paraffin. Sections were immunostained following the labeled streptavidin-biotin technique (LSAB2, Dako, Glostrup, Denmark). Antigen retrieval was performed by heating in 10 mmol/l, pH 6.0 citrate buffer in a pressure cooker. Sections were incubated with monoclonal antibodies against P-Selectin (clone 1E3, 1:100, Dako, Glostrup, Denmark) and CD31 (a marker of endothelial cells) (clone JC70A, 1:60, Dako, Glostrup, Denmark). The development was carried out using diaminobenzidine and 3% hydrogen peroxide. Slides were evaluated by light microscopy. The percentage of stained lumina for P-Selectin out of a total of CD31 positive vessels (P-S/CD31 index) was calculated at both edges in every specimen, under X200 magnification using a BX40 Olympus microscope (Tokyo, Japan). The ratio between both indices (injury and opposite edge) was calculated for every specimen.

Statistical analysis was performed using the independent samples *t*-test by the Sigma Stat Program (SPSS). A P value of <0.05 was considered statistically significant.

3. Results

All endothelial cells showed reactivity for CD31 (Fig. 1). A variable number of capillaries also stained for P-Selectin in all of the samples (Figs. 2 and 3).

P-S/CD31 index showed a great variation both at the wound edge and at the opposite margin in vital and post-

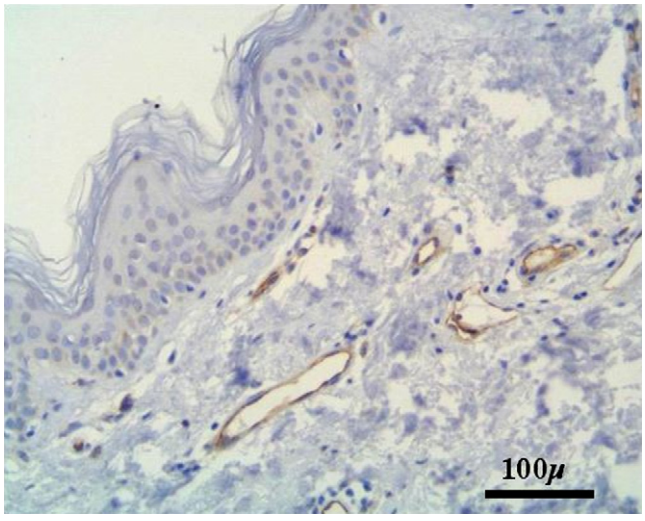

Fig. 1. CD31: endothelial cells of dermal capillaries are positive (Immunoperoxidase, ×200).

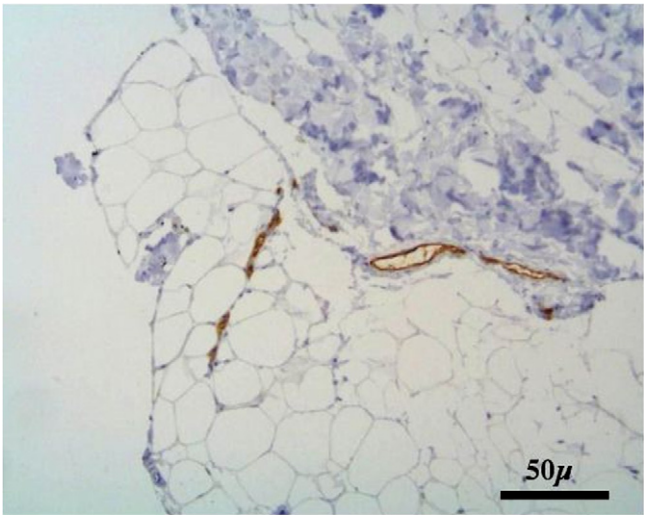

Fig. 2. P-Selectin: positive reaction of small blood vessels adjacent to vital wound edge (Immunoperoxidase, ×100).

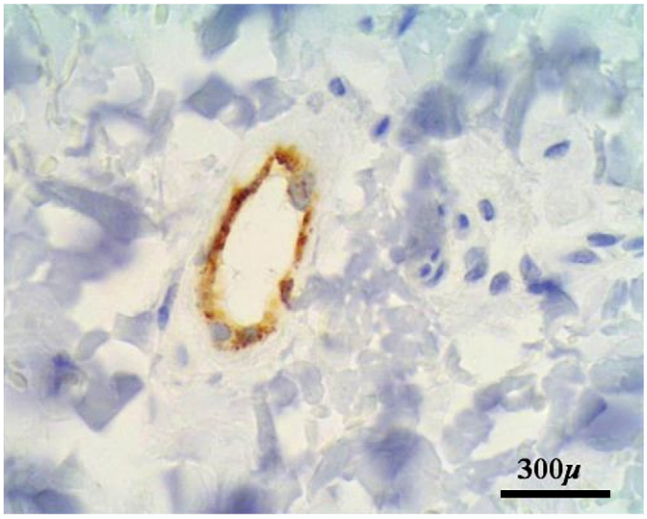

Fig. 3. P-Selectin: endothelial staining in a post-mortem injury (Immunoperoxidase, ×600).

Table 1
P-S/CD31 index (%)

	Vital samples	Post-mortem cases
Wound edge	10.7–71.4	22.5–69.2
Opposite margin	12.5–58.8	28–89.5
Ratio between both borders	0.37–1.77 (mean: 0.94)	0.76–1.9 (mean: 0.96)

Percentage of stained lumina for P-Selectin out of the total of CD31 positive vessels.

mortem cases (Table 1). No statistically significant differences were found ($p = 0.38$) (Table 1) when the ratio of both indices (injury/opposite margin) was determined for vital and post-mortem groups.

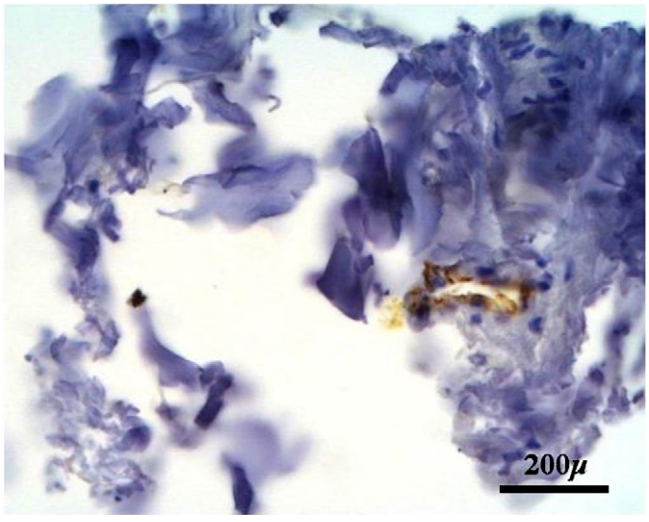

Fig. 4. P-Selectin in a specimen with autolysis/putrefaction: positive vessel in tissue with severe artifact (Immunoperoxidase, ×400).

Immunoreactivity for P-Selectin and CD31 persisted in the seven samples with a delayed fixation, but the staining was weak and showed background artifact (four cases) and considerable disruption of tissues (100%) (Fig. 4).

4. Discussion

The diagnosis of vitality of wounds is one of the central issues in daily forensic practice, in particular with regard to the differentiation between wounds inflicted shortly before and shortly after death.[17] Post-mortem injuries can occur accidentally (during handling of the corpse, in the necropsy, or caused by animals) but can also be intentional: trying to hide or suggest a crime, to simulate an accident or a suicide, or to occult the identity of a corpse.[18] Immunohistochemical analysis of a variety of tissular substructures, such as adhesion molecules, is considered the method of choice in modern investigations on vitality and wound age.[17]

Selectins are a family of cell–cell adhesion proteins involved in leukocyte–endothelial interactions. This group includes L-Selectin (also designated CD62L), E-Selectin (ELAM-1: endothelial leukocyte adhesion molecule-1, or CD62E), and P-Selectin (PADGEM: platelet activation-dependent granule membrane protein, or GMP-140: granular membrane protein-140, or CD62P).[19] Selectins initiate the rolling and, later, adhesion of leukocytes to the activated endothelium of microvasculature.[7,15,19] Endothelial P-Selectin acts as a receptor for neutrophils and monocytes, and its major ligand in polymorphonuclear leukocytes is PSGL-1 (P-Selectin Glycoprotein Ligand 1), also designated as CD162.[14]

As selectins are proteins involved in the early stages of the inflammatory process, their immunohistochemical analysis attracted the attention of Forensic Pathology, initially in the lung. Overexpression of P-Selectin has been observed in lungs after rapid death from hanging or intoxications, whereas a decrease of staining was found in cases of protracted death, such as pneumonia and sepsis.[20] Overexpression of E-Selectin could be found in lungs of patients with sepsis but not in those involving other causes of lung injury or other causes of death.[21]

A few reports on the use of immunohistochemistry to study these three selectins in cutaneous wounds have evaluated the percentage of positive vessels. Dressler et al. reported an overexpression of P- and E-Selectins in vital injuries, comparing some of the specimens with samples of intact skin from the same bodies, and found that 90% of the post-mortem induced wounds were negative.[7,16] A positive reaction was observed for P-Selectin after 3 min at the earliest age and 7 h at the latest. For E-Selectin, the reaction times were at 1 h and 17 days, respectively. The staining decreased after an interval of 12 h post-infliction. L-Selectin was only detected in leukocytes.[7,16]

The evaluation of P-Selectin estimated the number of positive vessels from a total of CD31 positive ones, and this

ratio P-S/CD31 may be considered an index of endothelial activation.[7]

In our series, the determination of this index has given widely variable results, both at the wound edge as well as at the opposite side. This fact seems unrelated to the age of the wound, and probably reflects the influence of unknown variables such as local tissue factors among others.

The comparison of immunoreactivity between both borders of a wound has proven useful for other early markers of vitality, such as fibronectin. A stained network extending from the wound edge to the adjacent dermis was observed in vital injuries when they were immunostained for fibronectin, whereas the opposite margin is negative.[5,6,9,10] With regard to P-Selectin, a comparison of the P-S/CD31 index between both edges of each specimen in vital and post-mortem wounds revealed no significant differences. The amount of tissue evaluated for each edge was possibly too little (approximately 1.1 mm from the border into the adjacent dermis), or perhaps the method of evaluation was too subjective. We have used single wound specimens in order to simplify the analysis of cases and to reproduce forensic casework. This method differs from those reports which compared the immunoreactivity of the injury with a sample of intact skin taken from a different location of the same body.[7,16]

The immunoreactivity of the vascular endothelium for P-Selectin cannot be considered a specific marker of vitality. Our post-mortem wounds, which were obtained in the first hours following death (mean: 6 h), have shown immunostaining as if it were a supravital phenomenon. This expression of P-Selectin could be correlated with prior descriptions of intravascular accumulation of white blood cells and even migration and tissue infiltration that may be induced post-mortem during the supravital period.[3,22,23]

Although autolysis and putrefaction have not eliminated immunostaining for P-Selectin and CD31, microscopic evaluation becomes difficult and, in some cases, impossible. Poor fixation may cause the decomposition of proteins with an alteration of their antigenic structure, causing reduced reactivity or enhanced background staining.[24,25] These artifacts of the immunohistochemical technique, in addition to those directly produced by autolysis and putrefaction (destructuring, fragmentation of tissues) make the histological interpretation of these cases very difficult, and the quantitative evaluation, almost impossible. This should be kept in mind since such circumstances are not rare in forensic casework. In general our data have pointed out the difficulties to apply immunohistochemistry to the diagnosis of vitality in real-life cases. Not only immunohistochemical techniques are not always accessible but also may have problems of sensitivity and specificity as seen in previous reports.[8–10] The results are dependent on fixation of the tissue, method of antigen retrieval and chosen antibody, and interpretation may be difficult or subjective.[17] These factors explain the disparity of results in the literature, and it has been recommended when evaluating a case to consider only positive reactions and to analyze more than one marker.[6,17] With regard to selectins there are only a few reports[7,16] and their application seems to be difficult in practice.

In conclusion, the analysis of P-Selectin/CD31 immunoreactivity in skin wounds was not useful for the diagnosis of vitality when evaluating both edges for each specimen. Moreover, P-Selectin has been detected in post-mortem injuries and it is not specific to vital injuries. Autolysis and putrefaction do not eliminate immunoreactivity, but the presence of artifacts is of such importance that it greatly impedes microscopic evaluation. A limitation of our study could be that our post-mortem samples may not represent real-life cases because the injuries were introduced to post-mortem tissue, rather than inflicted in antemortem tissue and studied in post-mortem samples, and complementary experiments on this type of case material should be undertaken.

Acknowledgments

We are thankful to Ms. Carolina Gómez de María and Dr. Iosu Antón for their cooperation.

References

1. Betz P. Histological and enzyme histochemical parameters for the age estimation of human skin wounds. *Int J Legal Med* 1994;**107**:60–8.
2. Ohshima T. Forensic wound examination. *Forensic Sci Int* 2000;**113**:153–64.
3. Madea B. Importance of supravitality in forensic medicine. *Forensic Sci Int* 1994;**69**:221–41.
4. Viljanto J, Penttinen R, Raekallio J. Fibronectin in early phases of wound healing in children. *Acta Clin Scand* 1981;**47**:7–13.
5. Betz P, Nerlich A, Wilske J, Tübel J, Wiest I, Penning R, et al. Immunohistochemical localisation of fibronectin as a tool for the age determination of human skin wounds. *Int J Legal Med* 1992;**105**:21–6.
6. Betz P. Immunohistochemical parameters for the age estimation of human skin wounds: a review. *Am J Forensic Med Pathol* 1995;**16**:203–9.
7. Dressler J, Bachmann L, Koch R, Müller E. Enhanced expression of selectins in human skin wounds. *Int J Legal Med* 1998;**112**:39–44.
8. Grellner W, Dimmeler S, Madea B. Immunohistochemical detection of fibronectin in postmortem incised wounds of porcine skin. *Forensic Sci Int* 1998;**97**:109–16.
9. Ortiz-Rey JA, Suárez-Peñaranda JM, Da Silva EA, Muñoz JI, San Miguel-Fraile P, De la Fuente-Buceta, et al. Immunohistochemical detection of fibronectin and tenascin in incised human skin injuries. *Forensic Sci Int* 2002;**126**:118–22.
10. Ortiz-Rey JA, Suárez-Peñaranda JM, Muñoz-Barús JI, Álvarez C, San Miguel P, Rodríguez Calvo MS, et al. Expression of fibronectin and tenascin as a demonstration of vital reaction in rat skin and muscle. *Int J Legal Med* 2003;**117**:356–60.
11. McEver RP, Martin MN. A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. *J Biol Chem* 1984;**259**:9799–804.
12. Stenberg PE, McEver RP, Shuman MA, Jacques YV, Bainton DF. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J Cell Biol* 1985;**101**:880–6.
13. Johnston GI, Cook RG, McEver RP. Cloning of GMP-140, a granule membrane protein of platelets and endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. *Cell* 1989;**56**:1033–44.

14. McEver RP, Moore KL, Cummings RD. Leukocyte trafficking mediated by selectin-carbohydrate interactions. *J Biol Chem* 1995;**270**:11025–8.
15. Lorant DE, Topham MK, Whatley RE, McEver RP, McIntyre TM, Prescott SM, et al. Inflammatory roles of P-Selectin. *J Clin Invest* 1993;**92**:559–70.
16. Dressler J, Bachmann L, Strejc P, Koch R, Müller E. Expression of adhesion molecules in skin wounds: diagnostic value in legal medicine. *Forensic Sci Int* 2000;**113**:173–6.
17. Grellner W, Madea B. Demands on scientific studies: vitality of wounds and wound age estimation. *Forensic Sci Int* 2007;**165**:150–4.
18. Hernández Cueto C. Diagnóstico diferencial entre lesiones vitales y postmortales. In: Villanueva Cañadas E, editor. *Medicina legal y toxicología Gisbert Calabuig*. 6th ed. Barcelona: Masson; 2004. p. 351–9.
19. Rosen SD. Selectins. In: Kreis T, Vale R, editors. *Guidebook to the extracellular matrix and adhesion proteins*. 1st ed. New York: Oxford University Press; 1993. p. 168–71.
20. Ortmann C, Brinkmann B. The expression of P-Selectin in inflammatory and non-inflammatory lung tissue. *Int J Legal Med* 1997;**110**:155–8.
21. Tsokos M, Fehlauer F, Püschel K. Immunohistochemical expression of E-Selectin in sepsis-induced lung injury. *Int J Legal Med* 2000;**113**:338–42.
22. Ali TT. The role of white blood cells in post-mortem wounds. *Med Sci Law* 1988;**28**:100–6.
23. Grellner W, Madea B, Kruppenbacher JP, Dimmeler S. Interleukin-1α (IL-1α) and *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) as potential inducers of supravital chemotaxis. *Int J Legal Med* 1996;**109**:130–3.
24. Pelstring RJ, Allred DC, Esther RJ, Lampkin SR, Banks PM. Differential antigen preservation during tissue autolysis. *Hum Pathol* 1991;**22**:237–41.
25. Fieguth A, Kleemann WJ, von Wasielewski R, Werner M, Tröger HD. Influence of postmortem changes on immunohistochemical reactions in skin. *Int J Legal Med* 1997;**110**:18–21.